Retinoid Signaling Determines Germ Cell Fate in Mice

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than wild-type littermates in this assay. The effective toxin concentration in SV2B knockout mice was reduced by 60% as compared with that of wild-type mice (Fig. 4C). The remaining toxicity in SV2B$^{-/-}$ mice was probably mediated by SV2A and SV2C, which are not altered, and by gangliosides, which serve as low-affinity receptors.

By using the secretory vesicle protein SV2 as its protein receptor, BoNT/A attacks active neurons with high selectivity because active neurons expose more receptors during exocytosis. Because BoNT/A blocks vesicle exocytosis, theoretically, a successful BoNT/A entry event will shut down the subsequent exposure of more receptors, allowing toxin molecules to enter active synapses that have yet to be poisoned. It has been shown that BoNT/B uses synaptotagmins I and II to enter cells (*8*, *9*, *11*). BoNT/G has also been reported to bind synaptotagmins I and II (*31*). Thus, secretory vesicle recycling pathways may have been exploited by several BoNTs, thereby contributing to the efficiency of this class of toxins.

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Retinoid Signaling Determines Germ Cell Fate in Mice

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Germ cells in the mouse embryo can develop as oocytes or spermatogonia, depending on molecular cues that have not been identified. We found that retinoic acid, produced by mesonephroi of both sexes, causes germ cells in the ovary to enter meiosis and inititate oogenesis. Meiosis is retarded in the fetal testis by the action of the retinoid-degrading enzyme CYP26B1, ultimately leading to spermatogenesis. In testes of *Cyp26b1*-knockout mouse embryos, germ cells enter meiosis precociously, as if in a normal ovary. Thus, precise regulation of retinoid levels during fetal gonad development provides the molecular control mechanism that specifies germ cell fate.

The ability to generate haploid gametes by meiosis is a unique property of germ cells and is critical for sexual reproduction. Whether germ cells develop as oocytes or spermatogonia depends on the time at which they enter meiosis: If meiosis begins during fetal development, as occurs in the mouse ovary around 13.5 days postcoitum (dpc), oogenesis is triggered, whereas germ cells that delay the onset of meiosis until after birth, as occurs in the testis, adopt a spermatogenic fate (*1*). It is widely believed that fetal germ cells are intrinsically programmed to enter meiosis and initiate oogenesis unless specifically prevented from doing so by a putative "meiosis-inhibiting factor" (*2*). However, such a substance has not been identified in vivo, and the molecular mechanisms regulating entry into meiosis in the fetal ovary but not in the fetal testis are unclear.

We conducted an expression screen designed to identify genes expressed sex-specifically during mouse gonadogenesis (*3*). One of these, *Cyp26b1*, was initially expressed in gonads of both sexes but became strikingly male-specific by 12.5 dpc (Fig. 1, A to C). Section in situ hybridization revealed that *Cyp26b1* is expressed in nascent testis cords (Fig. 1D). Because these contain only two cell types, Sertoli and germ cells, and expression was maintained in W^e/W^e mutant gonads that lack germ cells (*4*) (Fig. 1E), it is clear that Sertoli cells are responsible for *Cyp26b1* expression. *Cyp26b1* was also expressed in some unidentified interstitial cells (Fig. 1D). By using quantitative reverse transcription polymerase chain reaction (RT-PCR), we found maximal expression of *Cyp26b1* at 13.5 dpc in male gonads (fig. S1).

Cyp26b1 encodes a P450 cytochrome enzyme that degrades the potent morphogen retinoic acid (RA) (*5*). RA regulates the de-

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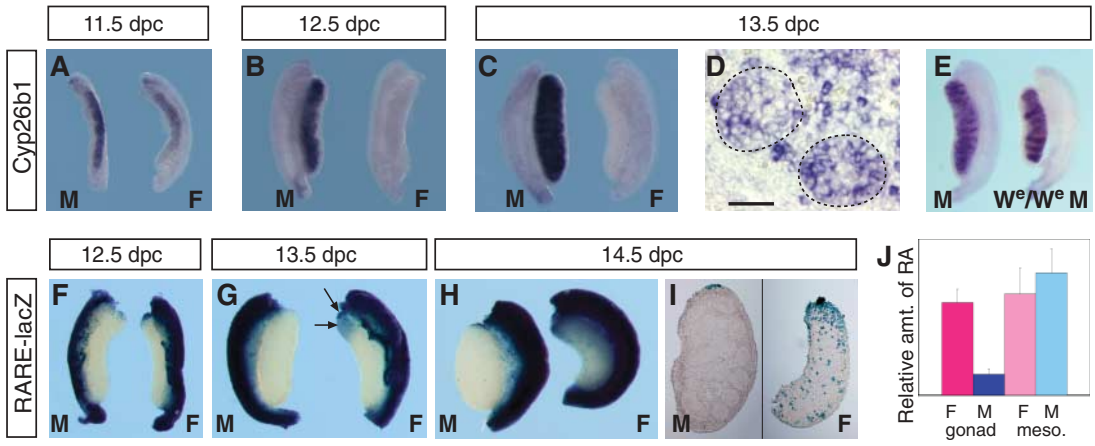

Fig. 1. CYP26B1 selectively removes RA from the developing testis. M, male; F, female. *Cyp26b1* expression in mouse UGRs at (**A**) 11.5 dpc, (**B**) 12.5 dpc, and (**C**) 13.5 dpc; (**D**) in a testis section at 13.5 dpc (two cords are outlined; scale bar indicates 50 μm); and (**E**) in W^e/W^e male UGRs at 13.5 dpc. *LacZ* reporter gene expression in RARE-LacZ transgenic mouse UGRs at (**F**) 12.5 dpc, (**G**) 13.5 dpc, and (**H**) 14.5 dpc and (**I**) in gonads separated from mesonephroi at 14.5 dpc (10-μm section). RA response is seen in the fetal ovary at 12.5, 13.5 [anterior, arrows in (G)], and 14.5 dpc (scattered throughout). (**J**) Quantitation of RA in extracts of 13.5-dpc gonads and mesonephroi using F9 RA indicator cells (arbitrary units). Error bars represent 1 SD (n = 3).

velopment of many organ systems, with local concentrations controlled by a balance of synthesis and degradation (*6*–*8*). The strong, male-specific expression of *Cyp26b1* in developing gonads is consistent with recent data implicating RA in the timing of meiotic initiation (*9*) and prompted us to investigate whether CYP26B1 might be the meiosis-inhibiting factor in male embryos.

To test for the presence of RA in the developing urogenital system, we used a mouse strain in which *lacZ* reporter gene expression is controlled by a RA response element (RARE-LacZ mice) (*10*). Strong *lacZ* staining was detected in mesonephroi of both sexes (Fig. 1, F to H). Little or no staining was found in developing testes (Fig. 1, F to I). In the ovary, *lacZ* staining, indicating a transcriptional response to endogenous RA, was seen in cells at the anterior pole at 12.5 (Fig. 1F), 13.5 (Fig. 1G), and 14.5 dpc (Fig. 1H) and also in scattered cells throughout the gonad at 14.5 dpc (Fig. 1, H and I). The relatively weak lacZ staining in these ovaries may reflect a technical limitation or an artifact of the transgenic indicator mouse line used (*10*), because quantitative measurements in gonad extracts revealed high levels of RA in the ovary (Fig. 1J). Extracts from male and female mesonephroi also contained high levels of RA, whereas testis extracts elicited a weak response (Fig. 1J).

To identify the source of the RA, we cultured explanted tissues on a lawn of RA-sensitive reporter cells overnight, removed them, and stained the reporter cells for LacZ expression; under these conditions, only sustained synthesis of RA (as opposed to residual RA content) elicits a response. Mesonephroi from 11.5 (Fig. 2A) and 12.5 (*11*) dpc embryos stimulated abundant *lacZ* gene activity in this assay, but male or female gonads at those stages did not, identifying the mesonephroi as sites of RA synthesis and hence the source of RA (Fig. 2B) in the developing urogenital system. Accordingly, we also found robust expression of *Aldh1a2*, encoding the major enzyme of RA synthesis in the mouse embryo (ALDH1A2, also called RALDH2) (*12*), in mesonephroi, but not gonads, of both sexes as early as 10.5 dpc, and persisting until at least 13.5 dpc (Fig. 2, C and D). These observations suggest a source-sink regulatory system (Fig. 2, E and F) controls sex-specific RA levels in fetal gonads.

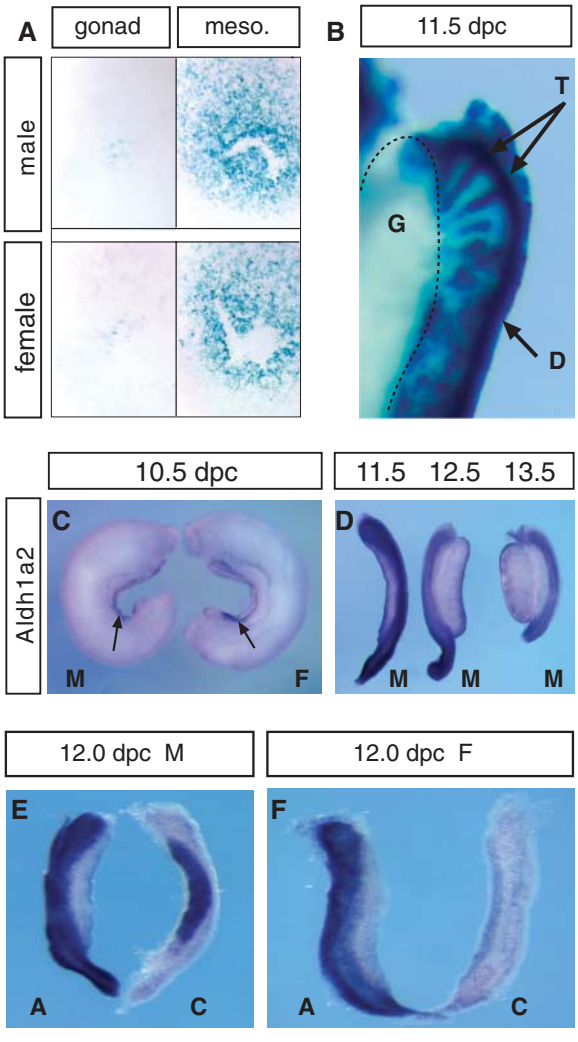

Fig. 2. The mesonephros is the source of RA in the developing urogenital system. (**A**) Detection of RA secreted over a 24-hour period by 11.5-dpc gonads and mesonephroi using F9 RA indicator cells. (**B**) *LacZ* reporter expression in mesonephric duct (D) and tubules (T) of an 11.5-dpc female RARE-LacZ UGR. The gonad (G) is outlined. (**C**) Expression of *Aldh1a2* in 10.5-dpc UGRs (attached to trunk of embryo; arrows indicate mesonephroi) and (**D**) in 11.5-, 12.5-, and 13.5-dpc male UGRs. Expression of *Aldh1a2* (A) and *Cyp26b1* (C) in pairs of UGRs from (**E**) male and (**F**) female embryos at 12.0 dpc.

We tested whether this system might underpin the oogenesis-spermatogenesis dichotomy by using urogenital ridge (UGR, mesonephros plus gonad complex) organ cultures and a variety of molecular markers of meiotic progression. These included *Scp3* (*Sycp3*), which encodes a component of the synaptonemal complex, and *Dmc1* (*Dmc1h*), which encodes a meiosis-specific recombinase, both robust markers of meiotic prophase (*13*, *14*). Addition of all-*trans* RA to male UGR cultures induced the premeiotic marker *Stra8* (*15*) and expression of *Scp3* and *Dmc1*, as measured by quantitative RT-PCR (Fig. 3A). Moreover, RA treatment suppressed the pluripotency marker *Oct4* (*Pou5f1*) (Fig. 3A) (*16*). In all RT-PCR experiments, results were normalized against expression levels of *mouse vasa homolog* (*Mvh*) (*17*), a marker of germ cells, to control for germ cell number. We also implanted RA-soaked beads into UGRs cultured on agar; in situ hybridization confirmed up-regulation of *Stra8* in gonads of both sexes (Fig. 3B). Hence, we conclude that exogenous RA stimulates the entry of germ cells into meiosis. This is likely to be a direct effect on germ cells, because we and others have found that RA receptors (RARs) and retinoid X receptors (RXRs) are expressed by germ cells at the relevant stages of development (*18*–*20*) (fig. S2).

To test the requirement for endogenous RA signaling, we exposed cultured gonads to the RA receptor antagonist AGN193109 (*21*). When female UGRs were cultured in the presence of AGN193109, the expression of *Stra8*, *Scp3*, and *Dmc1* was substantially decreased (Fig. 3, C and D) as was the number of meiotic chromosome figures in histological sections (Fig. 3E). Exposure to the antagonist also prevented the decrease in *Oct4* expression normally observed in fetal ovaries (Fig. 3C). These results confirm that RA provides the normal, endogenous signal for entry into meiosis in the fetal ovary.

Is CYP26B1 the meiosis-inhibiting factor in males? To address this question, we first exposed gonadal organ cultures to the cytochrome P450 inhibitor ketoconazole (*6*). In testis cultures, expression of *Scp3* and *Dmc1* increased to levels normally seen in ovary cultures, whereas expression of *Oct4* decreased (Fig. 4A). The effect on *Scp3* expression was confirmed by in situ hybridization (Fig. 4B). Moreover, meiotic figures were abundant among germ cells in treated testes but not found in untreated testes (Fig. 4C). The identity of CYP26B1 as the meiosis-inhibiting substance in males was confirmed in vivo by analysis of *Cyp26b1*-null mouse embryos (*7*). In these embryos, *Stra8* and *Scp3* were strongly up-regulated in XY gonads relative to wild-type and heterozygous XY littermate controls (Fig. 4, D and E). Interestingly, meiosis progressed earlier than normal in *Cyp26b1*-knockout ovaries (Fig. 4F). Therefore, early expression of *Cyp26b1* in female genital ridges (Fig. 1A) may ensure that germ cells in the female do not enter meiosis prematurely, given that *Aldh1a2* is expressed in mesonephroi of both sexes from as early as 10.5 dpc (Fig. 2C).

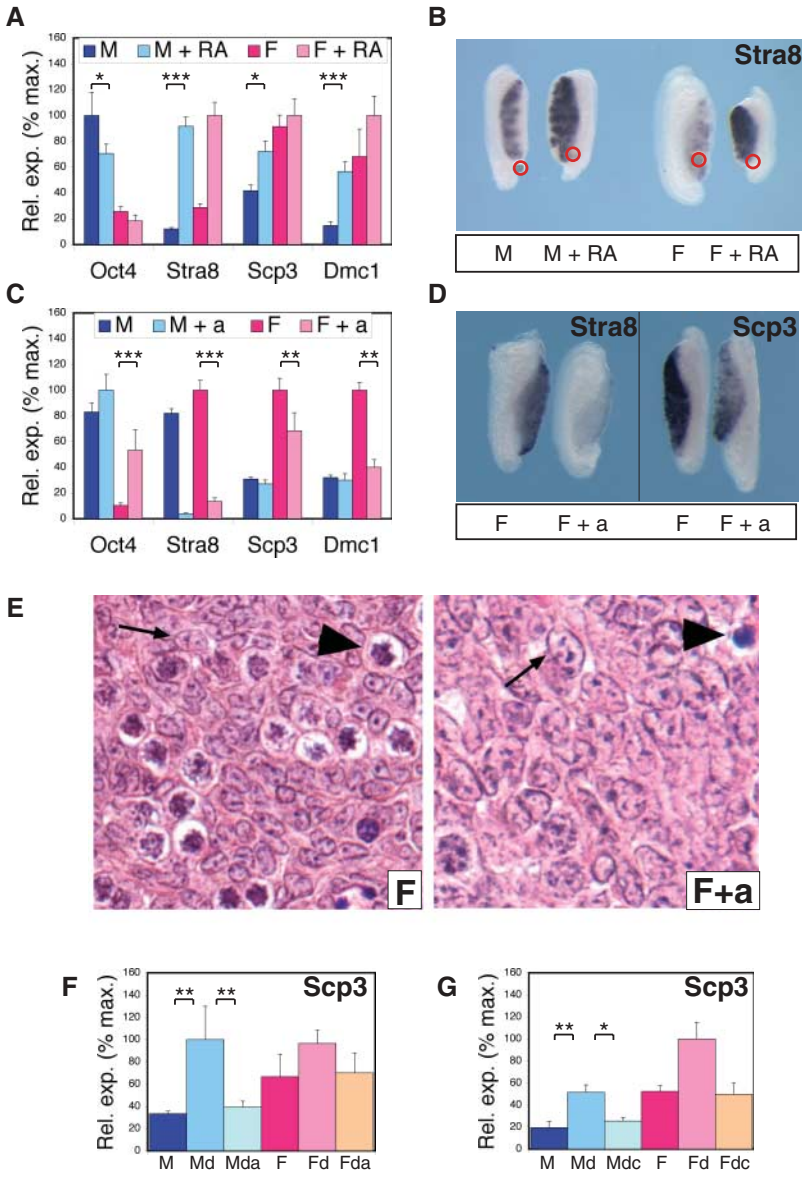

Fig. 3. Requirement for RA signaling in meiotic induction. Quantitative RT-PCR analysis of meiotic marker expression in UGR tissues treated with (**A**) all-trans RA or (**C**) RAR antagonist AGN193109. (**B**) In situ hybridization analysis of *Stra8* expression in UGRs after implantation with a bead (circled) soaked in all-trans RA. The samples shown were explanted at 12.5 dpc and cultured for 48 hours, which may explain the expression of *Stra8* in the control bead–implanted testis samples (fig. S3). (**D**) In situ hybridization analysis of *Stra8* and *Scp3* on female UGRs cultured with (F+A) or without (F) AGN193109. (**E**) Meiotic and nonmeiotic germ cells in 11.5 dpc–explanted plus 72 hour–cultured UGRs. Arrowheads, representative germ cells in meiotic prophase; arrow, representative nonmeiotic germ cells. Quantitation of *Scp3* expression after UGR dissociation-reassociation and culture with or without (**F**) AGN193109 or (**G**) citral. Bar graphs show the expression normalized to *Mvh*; error bars represent one standard deviation ($n = 3$). For (A) and (C), normalization to 18s rRNA was also carried out, with similar results (fig. S4). Expression is shown relative to maximal expression for the gene in question. Asterisks highlight the pertinent comparisons and indicate level of statistical significance (one asterisk, $P < 0.05$; two asterisks, $P < 0.01$; and three asterisks, $P < 0.0001$). a, AGN193109; d, dissociated-reaggregated; c, citral.

In previous studies (*2*), when 11.5-dpc male UGR cells were dissociated then reaggregated and cultured, germ cells entered meiosis, and it was postulated that destruction of the gonad architecture might disrupt produc-

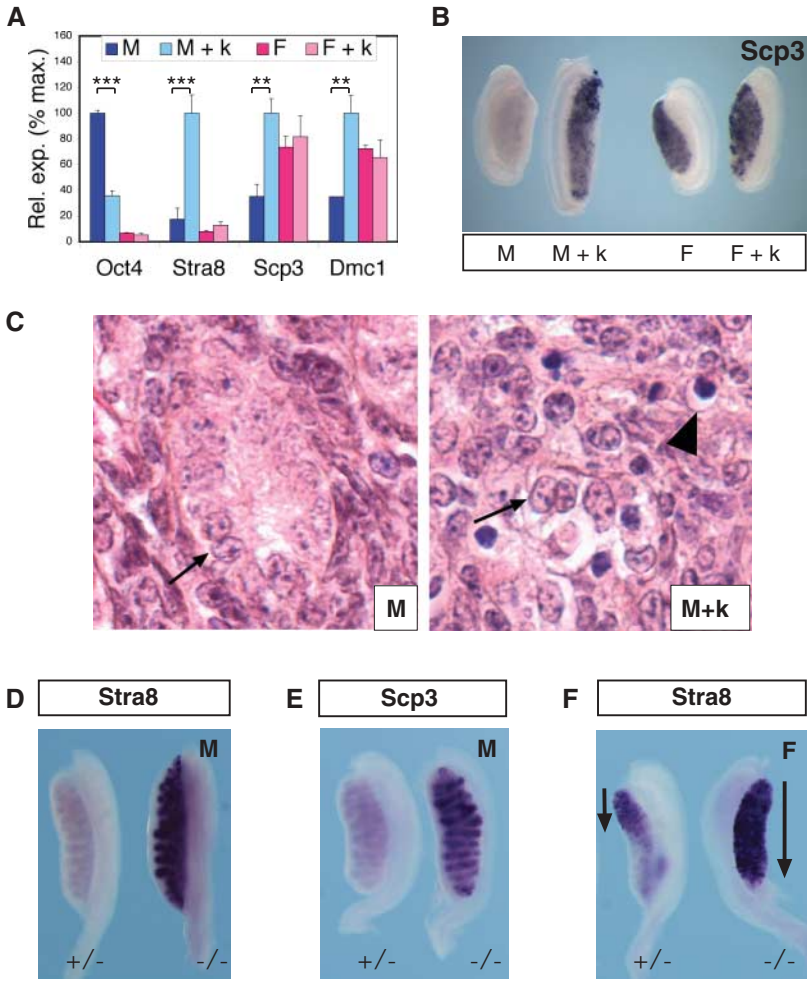

Fig. 4. CYP26B1 is the meiosis-inhibiting factor in male embryonic gonads. (**A**) Quantitative RT-PCR analysis of meiotic marker expression in UGR tissues treated with the P450 antagonist ketoconazole. (**B**) In situ hybridization analysis of *Scp3* expression in UGRs cultured with ketoconazole. (**C**) Meiotic and nonmeiotic germ cells in 11.5 dpc–explanted plus 72 hour–cultured UGRs. Details as in Fig. 3 legend; k, ketoconazole. (**D**) *Stra8* and (**E**) *Scp3* are strongly up-regulated in 13.5-dpc gonads of male embryos null for *Cyp26b1* (−/−) relative to heterozygous littermates (+/−). (**F**) *Stra8* is expressed in an expanded domain in 13.5-dpc gonads of female embryos null for *Cyp26b1*. Because meiosis occurs in an anterior-to-posterior wave in the developing ovary (*14*, *22*, *23*), meiosis is evidently more advanced in the knockout ovary (arrows). For each probe, four samples were analyzed for each genotype and representative samples are shown.

tion or activity of the meiosis-inhibitory factor. Our present data suggest that this treatment would place XY germ cells in close contact with RA-producing mesonephric cells and so induce meiosis. We tested this hypothesis by repeating these experiments in the presence of RAR antagonist AGN193109. Disassociation and reaggregation of untreated male UGRs at 11.5 dpc led to up-regulated expression of *Scp3* in XY germ cells (Fig. 3, F and G), consistent with published findings (*2*). However, when we included AGN193109 in aggregation cultures, *Scp3* was not induced (Fig. 3F). Similar results were obtained by using citral, an inhibitor of RA biosynthesis (*22*) (Fig. 3G). These results suggest that exposure of germ cells to RA-producing mesonephric cells was responsible for the induction of meiosis in the dissociated-reaggregated samples.

Induction of meiosis by RA released from the mesonephros is consistent with the anterior-to-posterior wave of meiotic progression in the fetal ovary (*15*, *23*, *24*). We observed very strong RA-induced gene expression in the mesonephric tubules (Fig. 2B), which are adjacent to the anterior pole of the gonad and connect the mesonephric duct with the gonad at 11.5 dpc (*25*). These tubules likely allow influx of RA, or RA-producing cells, to the gonad, resulting in earlier entry into meiosis anteriorly. This hypothesis is supported by transplantation and co-culture studies suggesting that the ovarian rete (the region containing tubules connecting mesonephros and ovary) produces a diffusible meiosis-inducing substance (*26*, *27*). Furthermore, in ovotestes, those germ cells that enter meiosis tend to lie adjacent to the ovarian rete (*28*), and in fetal testes meiotic germ cells are occasionally observed at the anterior junction between testis and mesonephros (*23*). Our results also accord with observations that RA can accelerate entry of rat germ cells into meiotic prophase when treated in cell culture (*29*).

Recent observations (*9*) indicate that RA stimulates *Stra8* expression in germ cells of mouse fetal ovaries and suggest that cytochrome P450 activity, ascribed to peritubular myoid cells, antagonizes this effect. Our experiments show that (i) RA induces full-scale meiosis, as judged by chromosomal condensation and molecular markers that directly correlate with meiotic prophase; (ii) the mesonephric duct and tubules are the source of RA in the urogenital system; (iii) high levels of RA are present in the fetal ovary but not the testis; (iv) *Cyp26b1* is expressed in Sertoli cells of the developing testis; and (v) CYP26B1 holds the key to preventing oogenesis in males by retarding meiosis in vivo. Although the existence of a meiosis-inhibiting factor was postulated some years ago (*2*), our studies reveal that this factor this is not a secreted signaling molecule as predicted but instead an enzyme of retinoid metabolism. Our findings may be applicable to modulating human or animal fertility in vivo and production of functional gametes from germline stem cells in vitro.

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